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Ready-to-Eat Vegetables: Microbial Quality and Active Packaging Solutions

Laura Franzetti, Alida Musatti, Lucia Caldera, Manuela Rollini*

DeFENS, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria 2, 20133 Milano
Manuela.rollini@unimi.it

Ready-to-eat (RTE) vegetables belong to the convenience foods category: they offer features like freshness, commodity of use and good retention of nutritional qualities. Nevertheless, as the raw material is characterized by a high enzymatic content and water activity, it represents an excellent substrate for microorganisms. For these reason, microbial growth is a key factor in RTE product deterioration.
Total bacterial count (TBC) was taken as the most relevant index to define hygiene and quality. Lactic acid bacteria, yeasts and moulds were present only occasionally. TBC was found lower when the product is packed under modified atmosphere. Gram-negative aerobic rods are dominant in air-packaged products, whilst the presence of *Enterobacteriaceae* becomes important in salads packaged under Modified Atmosphere. *Pseudomonas fluorescens* was the most frequently found species among the aerobic isolates, whilst for the *Enterobacteriaceae* strains no dominant species was found.
These isolated strains were tested for their sensitivity against two natural antimicrobial compounds, carvacrol (essential oil, active in vapour form), and ethyl-lauroyl-arginate (LAE, water soluble). Sensitivity was compared with that evidenced for strains belonging to official collections. Near 60 % of the tested Gram negative bacteria were found sensitive to LAE at a concentration of 20 mg/L, and near 90 % at 50 mg/L. Instead, carvacrol antimicrobial activity seemed more strain specific and not evidenced against lactic acid bacteria (LAB). A higher antimicrobial effect was found when the two antimicrobials were used in association. This combined approach, effective both in vapour and liquid phase, can represent an innovative solution to increase shelf life of RTE products.

1. Introduction

Ready-to-eat (RTE) vegetables are fresh products subjected to minimal processing to preserve their freshness. Their production technique is very simple and does not imply severe treatments or the use of preservatives: they are peeled, washed, dried, cut and packed in sealed pouches or in trays wrapped with extensible film then marketed ready to eat without further handling from the user (Baur et al., 2005; Ragaert et al., 2007) For this reason RTE vegetables belong to the family of convenience foods that offer a number of included features (freshness, commodity of use and extended shelf life).
RTE products are easily damaged because in addition to the microbiological problems of fresh product there are those from processing. Therefore the stabilities of these products depend on the qualities of raw materials, the handling procedures, the packaging modality the storage conditions and above all the respect of cold chain during distribution (Gimenez et al., 2003; Caponigro et al., 2010).
Herbs and spices have been known for their antimicrobial activity, often associated with the essential oil fraction, mainly composed of terpenes and phenols. The safe use of herbs or spices and their components has led to their current status of being food-grade or Generally Recognized As Safe (GRAS) food ingredients. One of the essential oil components with antifungal and antibacterial effects is carvacrol, present in high amount in the essential oil fraction of oregano (60–74 %) and thyme (45 %) (Ultee et al., 1998). Thanks to its appropriate hydrophobicity, carvacrol can be accumulated in the cell membrane. Its hydrogen-bonding ability and its proton-release ability may induce conformational modification of the membrane resulting in the cell death (Ben Arfa et al., 2006).

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One of the most innovative antimicrobial agent is ethyl-Nα- dodecanoyl-L-arginate hydrochloride (LAE). It is a synthetically derivative of lauric acid, L-arginine and ethanol (Ruckman et al., 2004), which is notable for its antimicrobial effectiveness resulting from its chemical structure and surfactant properties (Higueras et al. 2013). LAE's antimicrobial properties are due to its action as cationic surfactant on cytoplasmic membrane and the outer membrane of Gram-negatives, and cell membrane and cytoplasm of Gram-positive denaturation proteins. These changes produce disturbances in membrane potential, resulting in cell growth inhibition and loss of viability (Luchansky et al., 2005). LAE is characterized by a high antimicrobial efficiency also against fungi and yeasts, with a low-dose application (Rodriguez, 2004). In addition, LAE has a low oil-water equilibrium partition coefficient (K_{OW} < 0.1), which means that it tends to concentrate in the aqueous phase, where most bacterial action occurs (Ruckman et al., 2004). On top of that, LAE shows chemical stability and antimicrobial activity in a range of pH 3-7 (Asker et al., 2011).
With respect to other antimicrobial compounds, LAE when ingested is rapidly metabolized to natural endogenous compounds present in the human diet (i.e. arginine, lauric acid and ornithine). This property gives LAE an important degree of security. To date, LAE has been classified as GRAS and the USDA (United States Department of Agriculture) has approved its use in meat and poultry products, but is currently not approved in RTE products (Theinsathid et al., 2012; WHO, 2009).
The aims of this study were (i) to assess the microbiological quality and microbiota in RTE vegetables, (ii) to determine the antimicrobial activity of LAE and carvacrol against several microbial isolates, evaluating the possibility of associating them to obtain a synergic effect. This combined approach, effective both in vapor and liquid phase, would represent an innovative solution to increase shelf life of RTE products.

2. Materials and methods

2.1 Isolation

Two Ready-to-use green leafy vegetables, packaged in air (S and L), two packaged under modified atmosphere (Lc and Fq) and julienne carrots (C) coming from GDO were analysed at the end of their shelf life. Ten grams of each sample were drawn, and homogenized with 90 mL of sterile 0.85 % tryptone salt solution in a sterile Stomacher bag, by means of Colworth 400 Stomacher for 2 min. Decimal progressive dilutions were prepared, and the following bacteriological determinations were carried out: Total bacterial count (TBC) by pour plates on Plate Count Agar (PCA, VWR, Germany), incubation at 30 °C for 48 h; Lactic acid bacteria (LAB) by pour plates on Man Rogosa Sharpe agar (MRS, VWR, Germany), incubation at 30 °C for 48-72 h under anaerobic condition (gas pack) and yeasts and moulds on MEA (Malt Extract Agar) having the following composition (g/L): glucose 20, malt extract 20, soy peptone 20, agar 15, pH 5.8, incubation at 25 °C for 5 days. All the colonies growth on the last dilution of PCA and MRS were isolated and after purification were classified at specie level by total sequencing of 16S rDNA.
Twenty-four bacterial strains isolated from these vegetables as well as six strains belonging to official collections were employed to assess antimicrobial activity of LAE and carvacrol.

2.2 Determination of LAE antimicrobial activity

Minimum inhibitory concentration (MIC) of LAE was determined by inoculating a microbial cell suspension (OD 600 nm: 0.400, 100 µL) in 10 mL of culture medium with different amounts of LAE (0, 5, 10, 20, 50 and 100 mg/L) and incubated for 24–48 h at 30 °C. Turbidity was determined employing a spectrophotometer (Jenway, UK). MIC was determined as the lowest LAE concentration able to inhibit microbial growth.

2.3 Determination of carvacrol antimicrobial activity

To determine carvacrol antimicrobial activity, cell suspensions were prepared by serial dilutions and four dots of each suspension (3 µL) were put on agar surface. Plates were then inserted inside an air-tight glass jar (0.5 L total capacity). Increasing quantities of carvacrol (8.5, 25, 50, 100, 150 µL) were deposited on sterilized filter papers, that were inserted in the jar; considering the jar volume, the obtained carvacrol concentrations were 17, 50, 100, 200 and 300 µL/L. For each tested strain, a positive control sample was prepared by incubating the inoculated plate in a jar without carvacrol. Strains were incubated for 24-48 h at 30 °C. These tests were based on the presence or absence of growth, which was assessed visually. The Minimum Inhibitory Concentration (MIC) was defined as the minimal concentration of carvacrol required to completely inhibit bacterial growth.

2.4 Evaluation of LAE-carvacrol combination effect

The determination was performed in solid medium as follows: 12 mL TSA or MRS agar were pour plated in presence of the previously described serial LAE dilutions, made up in sterile Milli-Q water. Solidified culture media were inoculated with 3 µL of cell suspension (OD 600 nm: 0.400). Plates were then placed inside air-

tight glass jars, containing sterilized filter papers added with carvacrol. Samples were incubated at 30 °C for up to 48 h.
The Fractional Inhibitory Concentration (FIC) indices were calculated as reported by Turgis et al. (2012), by adding the FIC values of antimicrobial compounds (a) and (b) (FICa + FICb). The FICa and FICb values represented the lowest concentrations of LAE and carvacrol that caused the inhibition of bacterial growth in the combination tests. Calculations were performed as follows:
FICa = (MICa combined / MICa alone)
FICb = (MICb combined / MICb alone)
FIC = FICa + FICb
For interpretation of the results, FIC≤0.5 assigned as a synergistic effect, 0.5≤FIC≥1 represented as an additive affect, 1≤FIC≥4 represented as no interactive effect and FIC≥4 antagonistic effect between the two tested antimicrobials.

2.5 Statistical analysis

All the experiments were performed in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) using SPSS, version 21.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Isolation

TBC was the most important index to define the quality of the products; in general it was lower in Lc and Fq, both packed under modified atmosphere; however the microbial distribution was different according to the product and the method of packaging (Caldera and Franzetti, 2014). In leafy products packaged in air, the aerobic forms belonging to the family of *Pseudomonadaceae (Pseudomonas* spp.) were dominant, in those packed in MAP, an increase of *Enterobacteriaceae*, facultative anaerobes rods, was observed. Lactic acid bacteria, negligible in leafy products packaged in air, increase in those packed in MAP, and become important in carrots (Figure 1). The identification showed that among *Pseudomonas* genus, *Pseudomonas fluorescens* was the dominant form in all products. Among *Enterobacteriaceae*, coming prevalent from modified atmosphere-packaged products, the most frequent species found was *Citrobacter freundii,* followed by *Rahnella aquatilis* and *Pantoea agglomerans*. Lactic acid bacteria were represented by different strains of heterofermentative obbligate cocci.

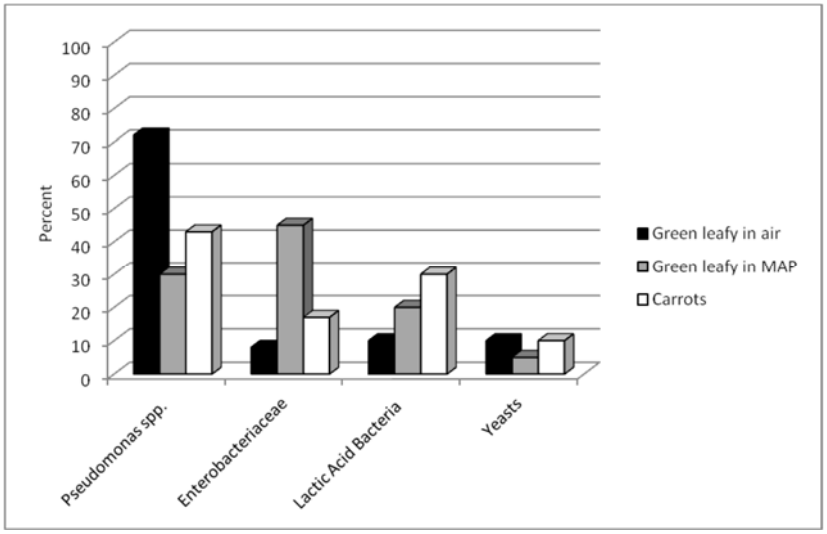

Figure 1 – Microbial percentage distribution in Ready-to-eat vegetables

3.2 LAE and carvacrol antimicrobial activity

MICs of the two antimicrobials applied singularly are reported in Table 1. As regards microorganisms isolated from RTE vegetables, near 60 % of the tested Gram negative bacteria were found sensitive to LAE at a concentration of 20 mg/L, and near 90 % at 50 mg/L. *Pseudomonas kilonensis* is the only Gram negative bacterium resistant at a concentration of 100 mg/L. As regard the Gram positives belonging to the species *Leuconostoc mesenteroides,* they were all very resistant at LAE higher than 100 mg/L.

Bacteria belonging to official collections were found very sensitive to LAE at 5-20 mg/L, the highest MIC being 50 mg/L against *Pseudomonas putida* ATCC 12633.

3.3 Antimicrobials combination

3.1 For the most resistant strains, trials were performed employing the two antimicrobials in association, and the positive results are reported in Table 2. The effect can be defined synergic against the strains *C. maltaromaticum* Fq80 and *L. mesenteroides* L43 and Lc6, with FIC ≤ 0.5; for all the other tested bacterial strains, the effect can be defined as additive (0.5 ≤ FIC ≥ 1.0). This combined approach, effective both in vapour and liquid phase, can represent an innovative solution to increase shelf life of RTE products. The use of antimicrobial agents in association would in principle provide a greater spectrum of activity, with increased antimicrobial action against pathogenic and/or spoilage organisms. Such a combination would act on different species of a mixed microbiota, or act on different metabolic elements within similar species or strains, resulting in an improved microbial control over the use of one antimicrobial agent alone (Santiesteban-Lopez et al., 2007). The antimicrobial features of food packaging materials can be achieved by different strategies: among others, the incorporation in the bulky polymer of migrating compounds, grafting of antimicrobial moieties, and immobilization of antimicrobial agents on the surface of the material in direct contact with the food are the most widely adopted routes. However, direct incorporation in the plastic polymer matrix is not a feasible approach when dealing with antimicrobials agents that are highly sensitive to package production conditions, i.e. carvacrol. To overcome these drawbacks, alternative production methods have recently been considered. In particular, coating technology can be proposed to its promising potential as a valid route to generate antimicrobial packaging materials containing LAE and carvacrol.

Table 1 - Minimum Inhibitory Concentration (MIC) of LAE and carvacrol in cultures of strains isolated either from RTE vegetables and official collections

Microorganism	MIC LAE (mg/L)	MIC carvacrol (µL/L)
Carnobacterium maltaromaticum Fq80	50	>300
Citrobacter freundii L55	20	300
Enterobacter cloacae Lc31	50	100
Enterobacter ludwigii Lc35	100	100
Leuconostoc mesenteroides C44	100	>300
Leuconostoc mesenteroides I6	>100	>300
Leuconostoc mesenteroides L43	100	>300
Leuconostoc mesenteroides Lc6	>100	>300
Leuconostoc mesenteroides S72	>100	>300
Pantoea ananatis Lc38	20	200
Pantoea agglomerans Lc34	20	100
Pseudomonas argentinensis S34	20	300
Pseudomonas fluorescens C20	20	200
Pseudomonas fluorescens Lc 44	5	200
Pseudomonas fluorescens S8	5	100
Pseudomonas kilonensis S9	>100	>300
Pseudomonas fragi Fq14	10	100
Pseudomonas koreensis C4	50	100
Rahnella aquatilis C5	50	200
Rahnella aquatilis C163	20	17
Rahnella aquatilis I35	5	50
Serratia fonticola S21	10	100
Serratia fonticola Fq65	50	100
Yersinia kristensenii I7	50	200
Escherichia coli CECT 434	10	200
Listeria innocua DSM 20649	20	200
Staphylococcus aureus ATCC 29213	5	100
Bacillus subtilis DSM 618	10	100
Pseudomonas putida ATCC 12633	50	>300
Sarcina lutea ATCC9341	5	>300

Coating technology has long since been used to improve especially barrier properties (e.g., against gases and moisture) and mechanical performance (e.g., coatings enabling the protection of the printed side of the package. Coatings can pave the way for new patterns to be created for the next generation of active packaging systems. The main benefit arising from this choice lies in the fact that coatings can be used as an additional layer to embed and release the active compounds, with no additional functions (e.g., barrier, optical, mechanical properties), which are still guaranteed by the plastic substrate (Chen et al., 2012).

Table 2 - Minimum Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration (FIC) indices of LAE and carvacrol employed in association.

Microorganism	MIC LAE (mg/L)	MIC carvacrol (µL/L)	MIC LAE + carvacrol (mg/L - µL/L)	FIC*
C. maltaromaticum Fq80	50	>300	20 - 8	0.22
Citrobacter freundii L55	20	300	2.5 - 150	0.63
Enterobacter cloacae Lc31	50	100	20 - 50	0.9
Enterobacter ludwigii Lc35	100	100	100 - 50	1.0
Leuconostoc mesenteroides L43	100	>300	50 - 8	0.5
Leuconostoc mesenteroides Lc6	>100	>300	100 - 25	0.5
Pantoea agglomerans Lc34	20	100	10 - 25	0.75
Serratia fonticola Fq65	50	100	100 – 25	0.75
Escherichia coli CECT 434	10	200	5 – 50	0.75
Listeria innocua DSM 20649	20	200	10 – 50	0.75
S. aureus ATCC 29213	10	100	5 – 50	1.0
Bacillus subtilis DSM 618	10	100	5 – 50	1.0

* FIC: Fractional inhibitory concentration. FICa = (MICa combined/MICa alone), FICb = (MICb combined/MICb alone), FIC = FICa + FICb

4. Conclusions

TBC is the most important index influencing the quality of RTE vegetables, however its composition depends on vegetables and the packaging technique used. LAE and carvacrol have great potentials as antimicrobial compounds: LAE was found active against Gram-negatives isolated from RTE products. Carvacrol antimicrobial activity seemed more strain specific and not evidenced against lactic acid bacteria (LAB). For some of the tested strains, a higher antimicrobial effect was found when the two antimicrobials were used in association, and in some case their effect can be considered synergic. These results would allow the use of lower carvacrol concentrations thus reducing any undesirable organoleptic impact on food products.
This combined approach, effective both in vapour and liquid phase, can represent an innovative solution to increase shelf life of RTE vegetable products and can help developing new package solutions for food preservation.

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